



TITLE:

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CITATION:

TOKORO, Masahiko ...[et al]. <Original>Isolation and Identification of the Trail Pheromone of the Subterranean Termite *Reticulitermes speratus* (Kolbe) (Isoptera: Rhinotermitidae). Wood research : bulletin of the Wood Research Institute Kyoto University 1992, 78: 1-14

ISSUE DATE:

1992-01-31

URL:

<http://hdl.handle.net/2433/53267>

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Isolation and Identification of the Trail Pheromone of the Subterranean Termite *Reticulitermes speratus* (Kolbe) (Isoptera: Rhinotermitidae)*¹

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(Received August 31, 1991)

Abstract—Approximately 100,000 workers of the termite, *Reticulitermes speratus* (Kolbe) were extracted with *n*-hexane to isolate trail pheromone. The extract was purified by silica gel column chromatography, normal phase HPLC and gas chromatography. Its trail-following activity was coincidentally examined by bioassays. The complete chemical structure of the pheromone was determined as (Z,Z,E)-3,6,8-dodecatrien-1-ol (DTE-OH) by means of instrumental analyses in conjunction with several micro-chemical reactions. Sternal gland extracts also contained DTE-OH, when analyzed by capillary gas chromatography mass spectrometry high resolution selected ion monitoring (GC-MS-HR-SIM).

Key words: *Reticulitermes speratus*, subterranean termite, trail pheromone, (Z,Z,E)-3,6,8-dodecatrien-1-ol, GC-MS-HR-SIM

1. Introduction

Significance and importance of trail pheromones in termite was well documented by several investigators¹⁻¹³. In spite of much research on the behavioral aspects of trail-following, only a few trail pheromones were identified chemically¹⁴⁻¹⁶.

Matsumura *et al.*^{17,18} isolated a trail pheromone from whole body extracts of *Reticulitermes virginicus* (Banks) (Rhinotermitidae). The amount of the isolated pheromone was too small to allow determination of the complete chemical structure of the pheromone. However, they found that a substance isolated from wood infected by a brown rot fungus, *Gloeophyllum trabeum* (Pers. ex. Fr.) Murr. was chemically similar to the trail pheromone of *R. virginicus*, and finally identified it was (Z,Z,E)-3,6,8-dodecatrien-1-ol (DTE-OH). They finally concluded that the chemical was identical with the trail pheromone of *R. virginicus*¹⁵.

*¹ A part of this report was presented at the 21st Annual Meeting of the International Research Group on Wood Preservation, Rotorua, New Zealand, May, 1990.

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It was later reported that DTE-OH elicited trail-following behavior for other species of the genus *Reticulitermes*, and *Coptotermes formosanus* Shiraki, belonging to the family Rhinotermitidae¹⁹⁻²²). Since the trail-following substance shows a weak species-specificity, DTE-OH appears to be common among the family Rhinotermitidae.

On the other hand, several investigators demonstrated that many of the analogs of DTE-OH could induce trail-following behavior for some Rhinotermitid termites^{19, 20, 23-25}).

The complete structure (positions and stereochemistry of the double bonds) of the trail pheromone DTE-OH was determined by comparison of active threshold level of synthetic compounds with that of the native pheromone¹⁵). Since the analogs of pheromone compounds possibly induce trail-following activity and the threshold level of this activity varies as a function of multiple components of these active substances, this approach remains questionable in the determination of the complete structure of the native trail pheromone²⁶). Moreover, Howard *et al.*²¹) evidenced that some of the Rhinotermitid termites could recognize their own extract trail. The finding might suggest that this species specificity could be caused by variation of trail pheromone. It is, therefore, important to examine whether Rhinotermitid termite actually secretes the DTE-OH or not.

In the present investigation, we tried to isolate and identify the trail pheromone from *R. speratus*, a common subterranean termite in Japan, for better understanding of the trail pheromone. Although we already reported the determination of geometric configuration of the trail pheromone²⁷), we did not refer to the isolation procedure and the biological techniques. In order to support the validity and reliability of the isolated trail pheromone, it is worthy to account for the isolation procedure and bioassay technique in detail. Furthermore, we also tried to demonstrate the coincidence in the chemical structure of the active substances extracted from both whole bodies and sternal glands with the aid of new analytical techniques.

2. Materials and Methods

2.1 Insects

Test insects used in the present investigation were obtained from two colonies of *Reticulitermes speratus* in the campus of Kyoto Institute of Technology. Individual termites were carefully taken out of the timber for extraction. The remaining termites from one colony with the timber were kept at $20 \pm 2^\circ\text{C}$ and $50 \pm 5\%$ relative humidity under moistened condition in the laboratory over three months until they were subjected to bioassay. In this investigation, we used undifferentiated pseudergates older than third instar as determined by the size of termite body (hereinafter referred to worker). The workers were placed in a Petri dish with a moistened filter paper for

one hour prior to bioassay.

2.2 Chemicals

(*Z,Z,E*)-3,6,8-dodecatrien-1-ol²⁸⁾ was supplied by Dr. H. Yamamoto (Nagoya University). (*Z,Z*)-3,6-dodecadien-1-ol and (*Z*)-3-dodecene-1-ol were supplied by Dr. G.D. Prestwich (State University of New York at Stony Brook). Isomers of 6,8-dodecadien-1-ol were supplied by Dr. T. Ando (Tokyo University of Agriculture and Technology). All other reagents were purchased from Nacalai Tesque INC. (NTI; Kyoto, Japan).

2.3 Bioassay method

A modification of the Prestwich *et al.*, Open-Field bioassay^{21, 25)} was used to establish threshold response levels for trail-following. As shown in Fig. 1, a dissolved sample (2 μ l) was streaked along a circle with a diameter of 4.7 cm (ca. 15 cm long) of pencil guide line (Fig. 1,c) drawn on fine quality clay coated paper, OK coat 135 K (Oji Paper Co. Ltd., Tokyo, Japan; Fig. 1,a) with a 5 μ l-micropipette. After evaporation of solvent, a plastic cylinder (1 cm \times 1.5 cm i.d.; Fig. 1,f) was placed on the paper at a left side of the test arena. Two openings (Fig. 1,d) of the cylinder directed the termite toward the test arena. A worker termite (Fig. 1,e) was then introduced into the cylinder, and a red colored Petri dish lid (1.5 cm \times 9 cm i.d.; Fig. 1,b) was placed above it in order to minimize the influence of air movements and light.

When a worker termite succeeded in moving along the sample streaked circle within two minutes, it was considered that a "basic activity" was induced. Nine rep-

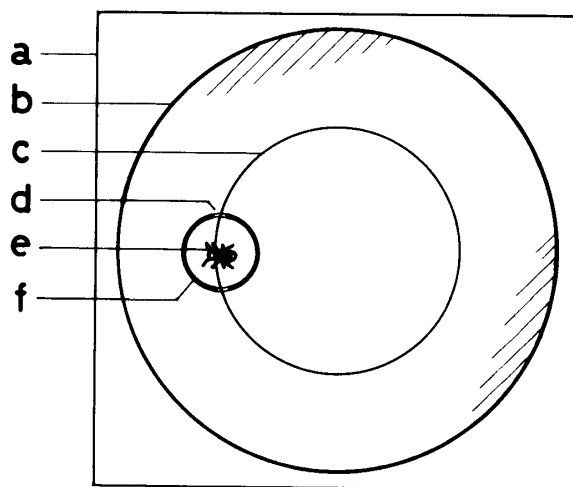


Fig. 1. Bioassay method.

Bioassays were modified Open-Field Bioassay (Howard *et al.*, 1976) with 2 μ l of test solution applied to a 15 cm circle.

a: fine quality clay coated paper, b: Petri dish lid, c: pencil guide line, d: opening, e: test worker, f: plastic cylinder

lications were done for each sample. When 1/3 or more of the test worker termites showed the basic activity, the sample was considered to have elicited a positive trail-following response. Ten fold dilution series of each sample were employed to determine the minimum effective worker equivalent (MEWE). The MEWE at which each sample elicited a positive activity was also determined for the more finely diluted solutions. Consequently, the relative strength of trail-following activity was compared on the basis of the MEWE. Bioassays were carried out at approximately 20°C and 50% relative humidity under the fluorescent lighting to estimate the trail-following activity. No termite was used for more than one bioassay in 24 hours.

2.4 Isolation

2.4.1 Extraction

Test individuals were placed on the moistened filter paper in a petri dish for about five hours before extraction, and soaked in *n*-hexane (Hx) for three days (ca. 100 ml per 100,000 individual workers). The extracts were then filtered and stored at -20°C until the next step. These operations were repeated to obtain extracts from approximately 150 g termite workers (ca. 100,000 in number).

On the other hand, one hundred fifth-sternites at which the sternal glands located were carefully dissected from the bodies of worker termites under a binocular stereoscopic microscope. The dissected fifth-sternites were soaked in 500 μ l of Hx for ten hours. As a control sample, one hundred of workers were soaked in 500 μ l of Hx for ten hours. Both of the extracts were used for capillary GC-MS-HR-SIM analysis.

2.4.2 Silica gel column chromatography

The whole body extracts were dried with anhydrous sodium sulfate for 1 hour, and concentrated by rotary vacuum evaporator until the yellowish crude lipid (ca. 3 g) was obtained. This crude lipid was first fractionated by silical gel column chromatography with Hx/ethyl acetate (EtOAc) and CH₃OH eluants. Silica gel (200 g; Silica Gel 60, 70-230 mesh; NTI) was packed into a glass column (1 m \times 5 cm i.d.) with the sample, and eluted with Hx/EtOAc, successively increasing the polarity, and with CH₃OH as shown in Fig. 2. Volume of eluants was one liter for each elution step, regardless of mixing ratios of Hx and EtOAc. It was finally separated into seven fractions.

2.4.3 Argentation silica gel column chromatography

The pooled active fractions were then fractionated by 20% (w/w) AgNO₃ silica gel chromatography with Hx/EtOAc in the similar manner as above (20 cm \times 7 mm i.d. glass column; AgNO₃ silica gel 1.5 g. Volume of eluants was 6 ml for each elution step. The active fractions were finally separated into 7 subfractions.

2.4.4 High performance liquid chromatography (HPLC)

Active fractions were fractionated by HPLC with Hx/EtOAc (7/3) and detected

by a UV detector at 234 nm. The analytical column was a normal phase Cosmosil 5SL (silica gel, 25 cm × 4.6 mm i.d., particle size 5 μ m; NTI) and guard column was a Cosmosil 10SL (5 cm × 4.6 cm i.d., silica gel, particle size 10 μ m; NTI). Flow rate of the eluent was 1.0 ml/min.

2.4.5 Gas chromatography (GC)

The final purification step was carried out by GC using a nonpolar column, followed by a polar column. Analytical conditions for each column were as follows: nonpolar column, 140°C (injection 250°C), carrier gas N₂ (40 cm/sec); polar column, 180°C (injection 250°C), carrier gas N₂ (25 ml/min). In both analyses, the outlet gas for each peak was isolated and the trail-following activity in response to it was determined. To obtain GC fractions for analysis, a glass tube cooled with dry ice in the middle portion, was indirectly connected to the metal head of the FID detector with a Teflon tube. The glass tube was rapidly rinsed with diethyl ether to extract the effluent for the later evaluation of trail-following activity.

2.5 Identification

The active GC peak was analyzed by capillary gas chromatography-mass spectrometry (GC-MS). The ion source temperature was 200°C, and the ionization energy was 70 eV. The oven temperature increased at a rate of 20°C/min over the range of 80–180°C, and samples were injected with a splitless injector in 2 μ l of Hx.

Complete structure determination of the trail pheromone was attempted by means of capillary GC-MS and capillary GC-Fourier transform infrared spectrometer (FTIR) analyses combined with microscale chemical reactions (acetylation, partial reduction, ozonolysis). The details should be referred to the previous paper²⁷⁾.

2.6 Capillary gas chromatography mass spectrometry High Resolution Selected Ion Monitoring (GC-MS-HR-SIM) analysis of the sternal gland extracts

The extracted components from sternal glands and whole termite bodies were condensed by an evaporator and analyzed by Capillary GC-MS-HR-SIM. The ion source temperature was 180°C, and ionization voltage was 70 eV. Samples were injected with a splitless injector in 0.5–1 μ l of EtOAc. The oven temperature increased at a rate of 30°C/min over the range of 120–210°C, and the injection temperature was 210°C. The resolution was approximately 5000. SIM monitors were set at m/z : 180.151 and m/z : 181.155, the former was precise molecular weight of DTE-OH and the latter was that of natural stable isotopes due to ¹³C.

2.7 Apparatus

The HPLC was a Model LC-5A (Shimadzu, Kyoto, Japan) pump connected to a model UVIDEK-100II detector (Jasco, Tokyo, Japan).

The GC employed a YANACO Model G180PF (Yanaco, Kyoto, Japan) equipped

with a flame ionization detector and a glass column (1.5 m \times 3 mm i.d.) packed with 5% Carbowax 20M-W-HP (100/120 mesh) (polar column; Hewlett-Packard (HP), Pennsylvania, USA) or a wide bore capillary column (10 m \times 0.53 mm i.d.) coated with methyl silicon (coating width 2.5 μ m) (nonpolar column; HP). Also used was a Shimadzu Model 7A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a fused-silica capillary column (CBP-20-25M-025, 25 m \times 0.25 mm i.d.; Shimadzu).

The GC-MS was a Model 5970 GC (HP) combined with a Model M-80B mass spectrometer (Hitachi, Ibaraki, Japan) equipped with a Model 0101 on-line data system (Hitachi). A fused-silica capillary column described above was used with this system.

The GC-MS for HR-SIM was a Model 5890 GC (HP) combined with a Model JMS-DX303HF (JEOL Ltd., Tokyo, Japan) equipped with a Model JMA-DA5000 mass data system (JEOL). A fused-silica capillary column (CBP-20-S25-050, 25

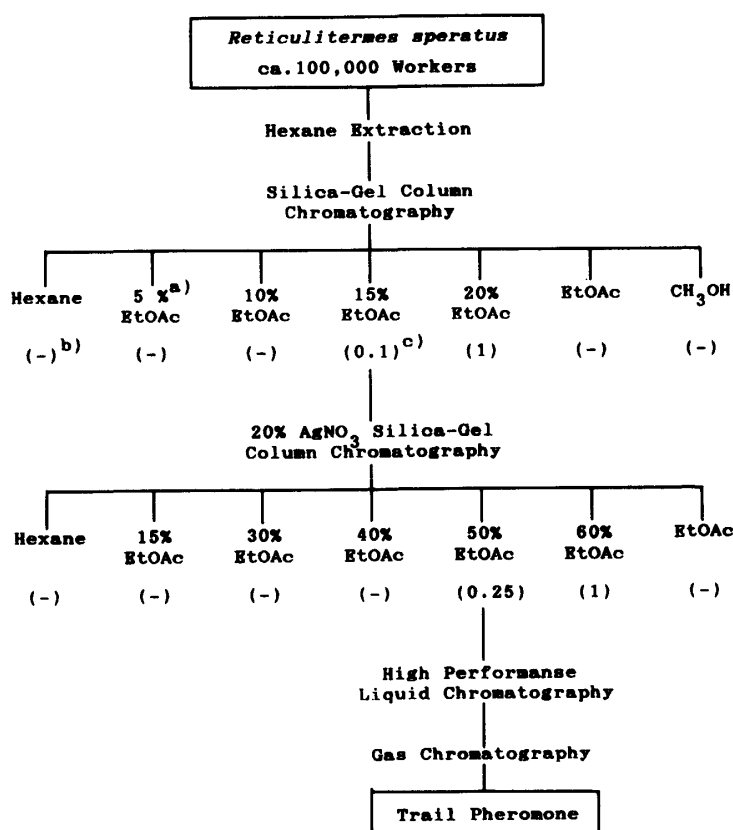


Fig. 2. The isolation procedure of the trail pheromone of *R. speratus*.
 a) Percentage by volume of ethyl acetate / *n*-hexane (%) as eluants
 b) No response or insufficient response for trail-following
 c) Value means trail-following activity as minimum effective worker equivalent

m \times 0.33 mm i.d.; Shimadzu) was additionally used with this system.

The GC-FTIR was a JEOL Model GCC-100GC-FTIR (JEOL) equipped with a capillary column (Carbowax 20M, 25 m \times 0.25 mm i.d.; HP) and with a high sensitivity MCT (Mercury Cadmium Telluride) detector (DET-101).

3. Results and Discussion

3.1 Isolation of the trail pheromone

Isolation procedure was shown in Fig. 2.

3.1.1 Results of bioassay with the crude hexane extracts

It is well known that sternal gland is the only secreting organ of the trail pheromone^{1,2,7,21,26-31}. Although a trail pheromone should be isolated through extraction only from the sternal gland or its secretion^{8,11,13,29}, it seems difficult to obtain a sufficient amount of the target components in a short time. Therefore, whole bodies of the insects were extracted by soaking in a solvent without homogenization in the present investigation.

Results of bioassay with the crude hexane extracts were shown in Table 1. As previously investigated, trail-following activity was induced by the trail pheromone within a limited range of worker equivalent (WE), and termites showed little or no response outside of the range. *R. speratus* was no exception to this rule^{3,12}.

The hexane extracts exhibited remarkable activities ranging from 0.1 to 100 WE per fifteen centimeter trail (WE/15 cm). The MEWE was thus determined as

Table 1. Trail-following bioassay with whole body extracts and sternal gland extracts.^{a)}

Quantity (WE/15 cm) ^{c)}	Percentage of Trail-Following (%) ^{b)}	
	Whole Body Extracts	Sternal Gland Extracts
0.001	0.0	0.0
0.005	6.7	3.3
0.01	10.0	6.7
0.05	26.7	16.7
0.1	63.3	76.7
1	93.3	96.7
10	90.0	100.0
100	80.0	93.3
1000	10.0	NT ^{d)}

^{a)} Bioassays were modified Open-Field Bioassay (Howard *et al.*, 1976) with 5 μ l of test solution applied to a 15 cm circle.

^{b)} Percentage of workers trail-following per 30 workers.

^{c)} Worker equivalent of 5 μ l test solution applied to a 15 cm circle.

^{d)} Not tested.

0.1 WE/15 cm. Its activity was comparable to that of sternal gland extracts. The corpses after extraction were homogenized with diethyl ether and served for the measurement of trail following activity. The activity was less than one-fiftieth of the primary hexane extracts.

Matsumura *et al.*^{17,18)} indicated that fungus-infected wood which was preferably ingested by termites because of the decayed wood contained the trail-following substance. Resultantly, it still remained unsolved whether the trail-following activity derived from the decayed wood ingested into digestive organs of termites. However, the extracts of intestinal contents of test workers showed no trail following activity in the preliminary experiment this time.

Bromination and acetylation markedly lowered the trail-following activity of crude hexane extracts. These results suggested that the trail pheromone of the extracts might have double bonds and hydroxyl groups like as DTE-OH.

3.1.2 Results of bioassay after silica gel column chromatographies

The trail pheromone was eluted mainly in the range of 15–20% EtOAc/Hx on silical gel column chromatography (Fig. 2). Fractions with this property usually consist of alcohols, sterols, and fatty acids. This elution pattern was similar to that of earlier reports³²⁾.

Based on the bioassay results of the trail pheromone following chromatography

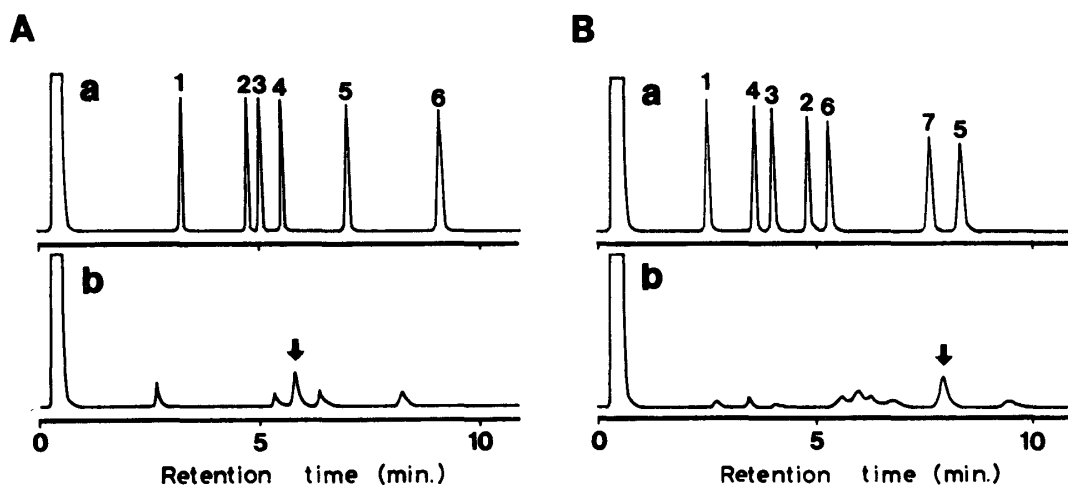


Fig. 3. Gas chromatograms using the nonpolar column (A) and the polar column (B).

(a) Authentic alcohols (Retention times (min) A & B);

- 1: $C_{11}H_{23}OH$ (tR.3.2 & 2.5), 2: (Z,Z)-3,6- $C_{12}H_{21}OH$ (tR.4.7 & 4.8),
- 3: (Z)-3- $C_{12}H_{23}OH$ (tR.5.0 & 4.0), 4: $C_{12}H_{25}OH$ (tR.5.5 & 3.6),
- 5: (E,E)-8,10- $C_{12}H_{21}OH$ (tR.7.0 & 8.3), 6: $C_{13}H_{27}OH$ (tR.9.1 & 5.3),
- 7: $C_{14}H_{29}OH$ (tR.7.6)

(b): The isolated trail pheromone; the arrows indicate the active peaks (tR. 5.8 & 7.9)

on 20% AgNO₃ silica gel, an active material was eluted mainly in the 50–60% EtOAc/Hx fractions (Fig. 2).

3.1.3 Results of HPLC preparation

The finding that the trail pheromone exhibited an absorption peak at 234 nm in UV spectroscopic analysis^{17, 18}, would suggest the presence of conjugated double bonds at defined by UV detector at 234 nm in the present study. The trail pheromone showed high activity in a peak at the time of retention around 2.6 minute, confirming the presence of conjugated double bonds.

3.1.4 Results of GC analyses

As shown in the results of GC analyses, the retention time of the trail pheromone peak was 5.8 minutes for a non-polar column (Fig. 3A,b), nearly identical with that of 1-dodecanol, and 7.9 minutes for a polar column (Fig. 3B,b), slightly less than that of 1-tetradecanol. The two different GC analyses suggested that this pheromone could be a twelve-carbon alcohol with conjugated double bonds.

3.2 Identification of the trail pheromone

3.2.1 The primary structure of the trail pheromone

Capillary GC-MS data are shown in Fig. 4. The total ion chromatogram of

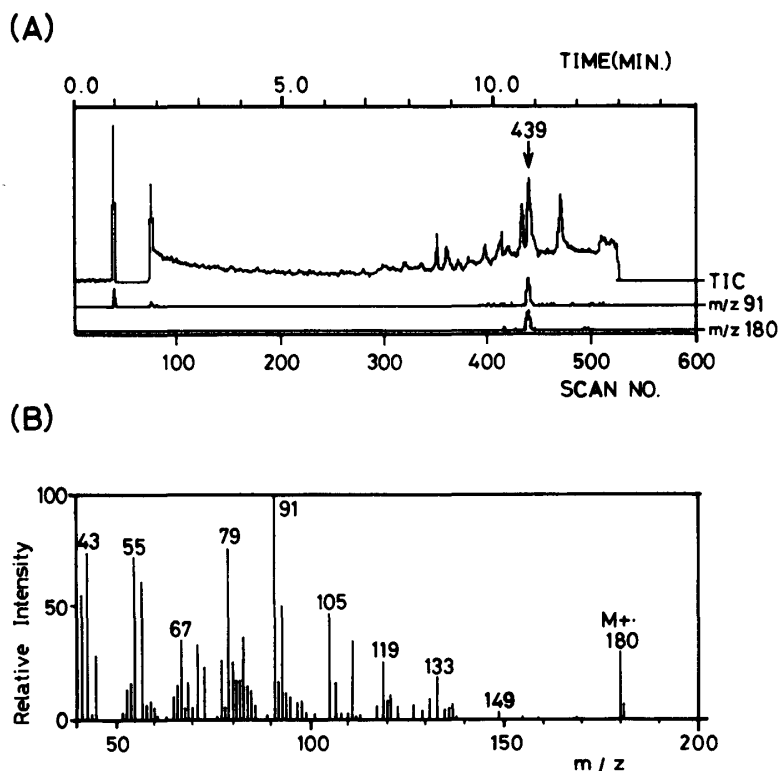


Fig. 4. Capillary GC-MS data of the isolated trail pheromone.
 (A) Total ion chromatograms and mass chromatograms (m/z : 180) of the isolated trail pheromone
 (B) Mass spectrum of the active peak Scan No. 439

the active fraction from HPLC gave the same pattern of the gas chromatogram. The electron impact (EI) mass spectrum of the trail pheromone showed the prominent molecular ion peak at m/z : 180 (M^+). The result suggested that the series of characteristic fragment ion peaks at m/z : 91, 105, 119, 133 might correspond to the increasing 14 mass units of the carbon skeleton of a trienyl alcohol following dehydration, which can be expressed as a general formula of C_nH_{2n-7} . These results thus showed that the substance had the typical pattern of a straight-chain primary alcohol with conjugated double bonds. The fact that alcohols with conjugated double bonds show prominent molecular ion peaks, while primary alcohols seldom show strong molecular ion peaks supports this conclusion. We confirmed that the primary structure of the trail pheromone of *Reticulitermes speratus* was straight-chained dodecatrien-1-ol containing conjugated double bonds. The amount of the trail pheromone per single worker termite was estimated as about 3 pg on the basis of external standard method of GC analysis.

3.2.2 The complete chemical structure of the trail pheromone

(a) Capillary GC-MS analysis of the trail pheromone acetate

The EI mass spectrum of the acetylated pheromone shows a molecular ion at m/z : 222 (M^+). It also showed a diagnostic ion at m/z : 162 ($M-60$) $^+$, which came from McLafferty rearrangement, and the series of prominent characteristic fragment ions *i.e.* m/z : 91, 105, 119, 133 were observed. These data indicated that the compound was corresponding to dodecatrienyl acetate with a conjugated double bond.

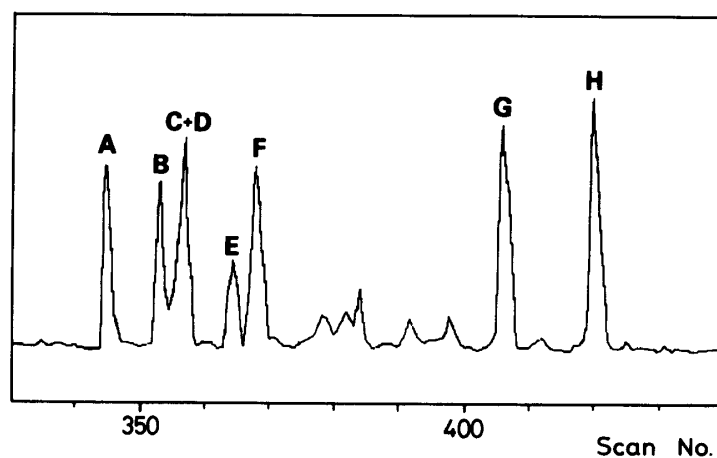


Fig. 5. Total ion chromatogram of the partially hydrogenated trail pheromone acetate.

A: Dodecanoic acetate ($C_{12}H_{25}OAc$); B, C+D: Dodecenyl acetate ($C_{12}H_{23}OAc$); E, F: Dodecadienyl acetate ($C_{12}H_{21}OAc$); G: Conjugated dodecadienyl acetate ($C_{12}H_{23}OAc$); H: Conjugated dodecatrienyl acetate ($C_{12}H_{19}OAc$)

(b) Capillary GC-MS analysis of the partially hydrogenated trail pheromone acetate

Capillary GC-MS analysis of the partially hydrogenated products of the acetylated pheromone showed seven peaks A, B, C+D, E, F, G and H (Fig. 5). A: saturated, B, C+D: monoenic, E, F, G: dienic and H: trienic respectively. The half height width of peak C+D indicated that it consisted of two monoenic compounds. The mass spectrum of peak G showed that it was a conjugated dienic one.

(c) Capillary GC-MS analysis of the partially hydrogenated trail pheromone acetate after ozonolysis

The total ion chromatogram of the ozonolysis products of the partially hydrogenated trail pheromone acetate showed major four peaks "W", "X", "Y" and "Z" (Scan No. 292, 149, 277, 397 respectively; Fig. 6). Component of the peak "W" was identified to unreacted dodecyl acetate by the retention time and mass spectrum.

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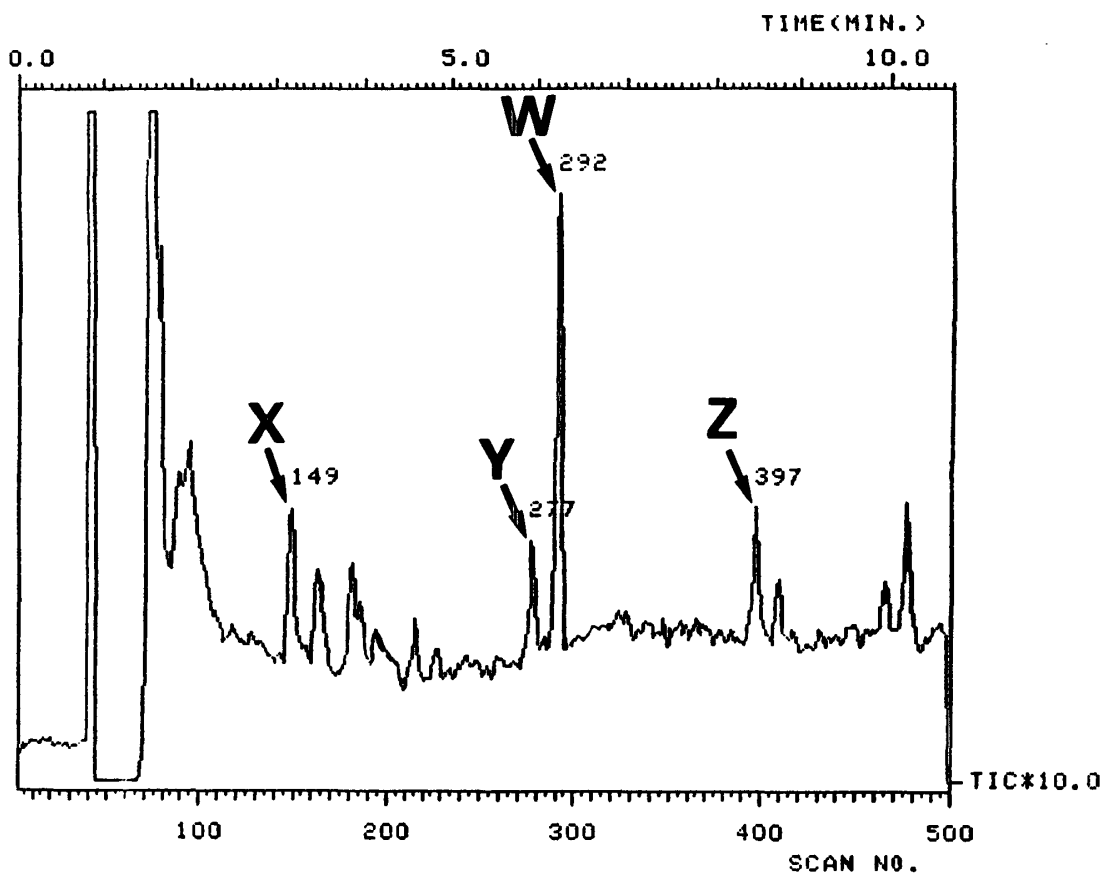


Fig. 6. Total ion chromatogram of the ozonolysis products of the partially hydrogenated trail pheromone acetate.

W: dodecyl acetate (Scan No. 292), X: 1-nonanal (Scan No. 149), Y: 6-acetoxy hexanal (Scan No. 277), Z: 8-acetoxy octanal (Scan No. 397)

Components of the peaks "X", "Y", "Z" were identified to 1-nonanal, 6-acetoxy hexanal and 8-acetoxy octanal by their retention times and mass spectra. Thus, the double bond positions of the trail pheromone were determined as 3, 6 and 8.

(d) Capillary GC-FTIR analysis of the partially hydrogenated trail pheromone acetate

The total absorbance monitoring (TAM) of GC-FTIR for partially hydrolyzed product also showed seven peaks. The FTIR spectrum of peaks C+D, E, G and H showed absorbance 940–980 cm^{-1} , indicating the presence of a *trans* carbon-carbon double bond in those compounds.

All the data supported that the configuration of the 8 position was *trans* and 3 and 6 were *cis*, and proved that the trail pheromone of the termite *Reticulitermes speratus* was identical with (Z,Z,E)-3,6,8-dodecatrien-1-ol (Fig. 7).



Fig. 7. The trail pheromone of the termite *Reticulitermes speratus* (Kolbe).

3.3 Capillary GC-MS-HR-SIM analysis of sternal gland extract

It is necessary to evidence the coincidence in the chemical structure of the active substances from both whole body extracts and sternal gland extracts. Otherwise, it can not be concluded whether the trail pheromone is true trail pheromone of *R. speratus* or not. Since the molecular weight of the trail pheromone is known theoretically, it is possible to detect the target component high-sensitively and high-selectively by means of capillary GC-MS-HR-SIM analysis.

Results of GC-MS-HR-SIM analyses shown in Fig. 8 demonstrated that the extracted components from sternal glands and whole termite bodies could be detected as sharp peaks of HR-SIM at m/z : 180.151 and m/z : 181.155, and that both of them could be high-selectively isolated from the impurities. The retention times of the both components were exactly the same as that of authentic DTE-OH. Therefore, the component of the sternal gland extracts corresponded to that of the whole body extracts, which was identical with DTE-OH.

On the basis of the present results of GC-MS-HR-SIM analysis, we concluded that the isolated substance from whole body was definitely the true trail pheromone of *R. speratus*.

Acknowledgements

The authors greatly appreciate Prof. H. Yamamoto for a generous gift of a authentic DTE-OH. We wish to thank generous gifts of (Z)-3-dodecen-1-ol and (Z,Z)-3,6-dodecadien-1-ol from Prof. Glenn D. Prestwich, State University of New

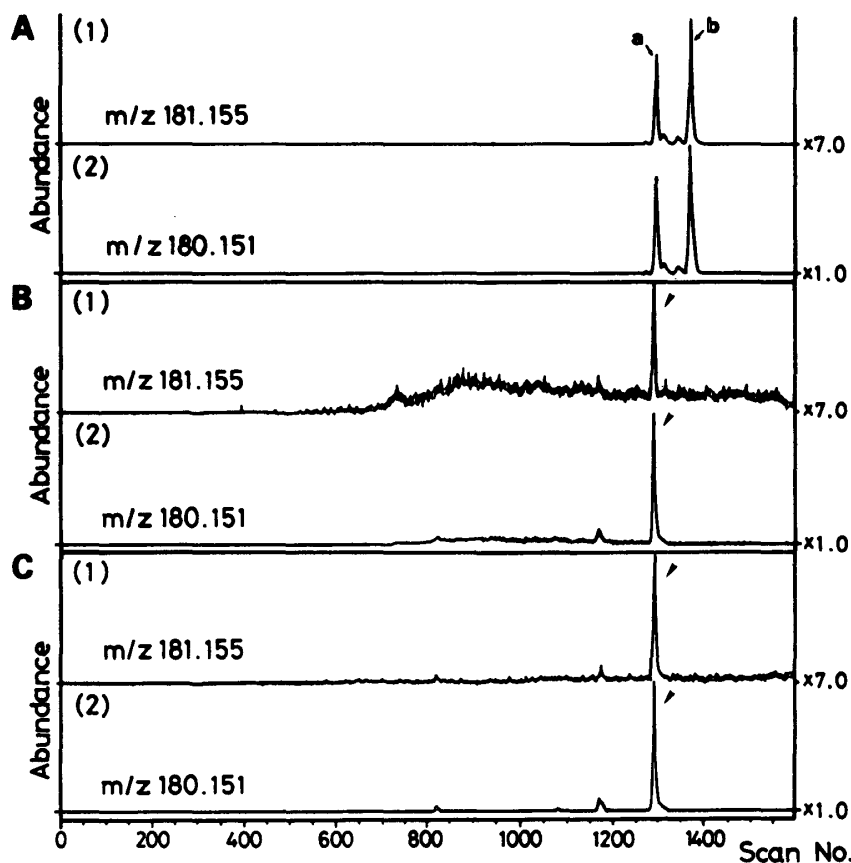


Fig. 8. Capillary GC-MS-HR-SIM data of the authentic dodecatrienol and the whole body extracts and the sternal gland extracts.

A: Authentic dodecatrienol isomers

a: (Z,Z,E)-3,6,8-C₁₂H₁₉OH: b: (Z,E,E)-3,6,8-C₁₂H₁₉OH

B: The whole body extracts

C: The sternal gland extracts

(1): HR-SIM chromatogram at *m/z*: 181.155

(2): HR-SIM chromatogram at *m/z*: 180.151

(The arrows indicated the active peaks)

York at Stony Brook. We wish to acknowledge a gift of the geometric isomers of 6,8-dodecadien-1-ol from Dr. T. Ando, Tokyo University of Agriculture and Technology. We wish to thank Dr. S. Ochiai and Dr. S. Ohgi of the SP group of JEOL. Ltd., for performing the capillary GC-FTIR. We also would like to thank Dr. T. Umezawa, Kyoto University, for performing the capillary GC-MS-HR-SIM. Sincere gratitude are also extended to Dr. B. McPherson, University of California Berkeley, for their valuable advice.

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